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Glucocorticoid receptor regulates accurate chromosome segregation and is associated with malignancy

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The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily, which controls programs regulating cell proliferation, differentiation, and apoptosis. We have identified an unexpected role for GR in mitosis. We discovered that specifically modified GR species accumulate at the mitotic spindle during mitosis in a distribution that overlaps with Aurora kinases. We found that Aurora A was required to mediate mitosis-driven GR phosphorylation, but not recruitment of GR to the spindle. GR was necessary for mitotic progression, with increased time to complete mitosis, frequency of mitotic aberrations, and death in mitosis observed following GR knockdown. Complementation studies revealed an essential role for the GR ligand-binding domain, but no clear requirement for ligand binding in regulating chromosome segregation. The GR N-terminal domain, and specifically phosphosites S203 and S211, were not required. Reduced GR expression results in a cell cycle phenotype, with isolated cells from mouse and human subjects showing changes in chromosome content over prolonged passage. Furthermore, GR haploinsufficient mice have an increased incidence of tumor formation, and, strikingly, these tumors are further depleted for GR, implying additional GR loss as a consequence of cell transformation. We identified reduced GR expression in a panel of human liver, lung, prostate, colon, and breast cancers. We therefore reveal an unexpected role for the GR in promoting accurate chromosome segregation during mitosis, which is causally linked to tumorigenesis, making GR an authentic tumor suppressor gene.

glucocorticoid receptor | mitosis | aneuploidy | DNA damage | cancer

Glucocorticoids (Gcs) act through the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily, and a ligand-activated transcription factor (1–4). The GR is ubiquitously expressed and regulates energy metabolism, immunity, and cell fate decisions. The quiescent GR resides in the cytoplasm in a complex with heat shock proteins and immunophilins, attached to the microtubule architecture of the cell in a heat shock protein 90-dependent manner (5). Ligand binding drives GR transformation, involving N-terminal phosphorylation on S203 and S211 and rapid translocation to the nucleus requiring attachment to dynein by heat shock protein 90, immunophilins, and dynamin (6). Once in the nucleus, GR binds directly to DNA to regulate transcription, or tethers to other DNA-bound transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and activator protein 1 (AP1), to regulate their function (7–10).

In mitosis, GR is phosphorylated on both S203 and S211, but in a ligand-independent manner (11), and more comprehensive phosphoproteomic analyses identify the presence of multiple N-terminal GR phosphoforms in purified mitotic spindle fractions (12). Although altered GR function in mitosis has been shown (11, 13, 14), the kinases responsible and cellular consequences

have not been defined. The role of GR in cancer has received little attention beyond acute lymphoblastic leukemia, in which GR is powerfully proapoptotic (15). However, GR inhibits expression of cyclin D and augments expression of cyclin-dependent protein kinase inhibitors p21 and p27 to effect cell cycle slowing or arrest in a cell type-specific manner (16).

We now define a role for GR in regulating mitosis using, at least in part, nontranscriptional mechanisms. Loss of GR results in a robust mitotic phenotype, with aberrant chromosome segregation, accumulation of chromosome complement defects, and death in mitosis, with the extent of cell survival or death varying by cell type and model. Moreover, GR haploinsufficient mice develop tumors with age. Analysis of common human cancers revealed frequent cell type-specific loss of GR expression, implying an important causal relationship between GR and malignant transformation or progression.

Results

We profiled the GR interactome (1,696 proteins) using immune enrichment and MS methods, and compared the list of GR interacting proteins with the mitotic spindle proteome (17). This

Significance

We have discovered a role for the glucocorticoid receptor (GR) in coordinating cell division. We find enrichment of GR to mitotic spindles and demonstrate that GR knockdown causes accumulation of mitotic defects, including delayed anaphase, ternary chromosome segregation, and death in mitosis. Mitotic GR function requires the ligand-binding domain but not ligand binding, revealing a nontranscriptional and ligand-independent mechanism of action. Analysis of GR haploinsufficient cells and tissues reveals increased aneuploidy and DNA damage, and mice show an increased incidence of tumors *in vivo*, with further GR loss within those incident tumors. We also identify reduced GR expression in several common human cancers, thereby implicating GR as a novel tumor suppressor gene.

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approach identified GR binding to complexes involving 478 of the 795 known protein components of the mitotic spindle (Fig. 1A and *SI Appendix*, Fig. S1), including both CENP-E and BUB3, which are required for spindle checkpoint function (18, 19). Analysis of purified mitotic spindle fractions by immunoblotting using specific phospho-GR and pan-GR antibodies revealed ^PS203 and ^PS211 modified GR species (Fig. 1B). The presence of multiple GR protein species on immunoblots is frequently seen, and may result from alternate protein isoforms or posttranslational modification. Phosphatase treatment did not result in loss of the additional bands, indicating that the mobility shift is not due to differential phosphorylation (*SI Appendix*, Fig. S2).

Immunofluorescence analysis confirmed GR localization to the mitotic spindle, with particular enrichment at the centrosomes in metaphase and anaphase cells (Fig. 1C and *SI Appendix*, Fig. S3). The mitotic distribution of GR to the spindle was also demonstrated using another antibody raised against a different GR epitope (*SI Appendix*, Fig. S4), was sensitive to knockdown with GR-specific siRNA (*SI Appendix*, Fig. S5), and was also seen using an epitope-tagged (HaloTag) GR (Fig. 1D and E). Analysis of Halo-GR-transfected cells showed a heterogeneous subcellular GR localization in asynchronous fixed cells, which visualizes both soluble and cytoskeletal-bound GR (total GR fraction; *SI Appendix*, Fig. S6A). A predominantly nuclear distribution was seen in permeabilized and washed-out cells following depletion of the soluble fraction (*SI Appendix*, Fig. S6A). The mitosis-specific distribution of Halo-GR was similar to that seen for endogenous GR (Fig. 1E and *SI Appendix*, Fig. S6B), reinforcing the specificity of GR recruitment to the mitotic spindle.

Previously, analysis revealed distinct subcellular distributions for ^PS203GR and ^PS211GR (20), and because both are enriched in mitosis, we tracked both phosphospecies through mitosis. We find ^PS203GR at the centrosomes, showing an overlapping distribution with the centrosome marker γ -tubulin (21) throughout mitosis (*SI Appendix*, Fig. S7). ^PS211GR also showed clear foci at the centrosomes, with additional signal overlapping with CREST-marked kinetochores (22) (*SI Appendix*, Fig. S8). To control for antibody specificity, we used multiple siRNA species,

which confirmed localization of total GR, and both ^PS203GR and ^PS211GR at the centrosome (*SI Appendix*, Figs. S5B, S7B, and S8B). Although GR is known to interact with the kinetochore protein Ska2 (23, 24), the distribution of ^PS211GR overlying the condensed chromosomes and kinetochores was not consistently lost with both GR siRNAs (*SI Appendix*, Figs. S8B and S9), suggesting that this immunoreactivity may be due to cross-reaction with another phosphorylated protein. Aligned surface plots of the three GR antibodies suggest robust enrichment of total GR and both GR phosphoforms to centrosomes (*SI Appendix*, Fig. S9).

The distribution of GR therefore overlaps with components of the microtubule organizing center, which is a site of Aurora kinase activity. The distribution of phospho-GR (both ^PS203GR and ^PS211GR) on the centrosome overlaps with Aurora A (*SI Appendix*, Fig. S10A). Therefore, we tested Aurora kinase inhibition, initially with tozasertib (which inhibits both Aurora A and Aurora B with a similar IC₅₀). Tozasertib virtually abolished mitotic GR phosphorylation on both S203 and S211 (*SI Appendix*, Fig. S10B).

Immunofluorescence of tozasertib-treated cells demonstrates that reduced ^PS203GR and ^PS211GR is not a consequence of mitotic exit but that mitotic cells (highlighted with asterisks in *SI Appendix*, Fig. S10C) are no longer enriched for GR phosphorylation relative to surrounding interphase cells. Inhibition of Aurora kinases disrupted normal spindle formation but did not prevent association of the GR with α -tubulin foci (*SI Appendix*, Fig. S10D), suggesting that phosphorylation of GR occurs subsequent to spindle targeting. If phosphorylation of GR at S203 and S211 occurred subsequent to spindle recruitment, then selective inhibition of Aurora A, but not Aurora B, would be predicted to abolish this modification because GR and Aurora A colocalize on the centrosome, and this effect was clearly seen (*SI Appendix*, Fig. S10E).

Identification of modified GR species at the mitotic spindle and regulation of GR modification downstream of Aurora A suggest a possible role for GR in mitosis. We next investigated whether knockdown of GR expression could affect chromosome segregation during mitosis in live cells using the expression of H2B-GFP as a chromosome marker. GR knockdown delayed the mean time from nuclear envelope breakdown to anaphase onset (from 76 to 102 min; Fig. 2A, *SI Appendix*, Fig. S11, and *Movies S1–S4*). The delay in completion of mitosis appeared to be due to defective chromosomal alignment/segregation because there was also a significant increase in abnormal anaphase cells and death in mitosis (Fig. 2B). Analysis of a cross-sectional population of targeted cells suggested that loss of GR expression resulted in a significant accumulation of cells in metaphase (Fig. 2C), further supporting a role for GR in facilitating metaphase/anaphase transition.

We also find increased numbers of cells with disorganized or multipolar spindles (Fig. 2D and *SI Appendix*, Fig. S11C) and micronuclei in interphase cells (Fig. 2E). These aberrant events were rescued by “adding back” siRNA-resistant GR (Fig. 2D and E), confirming the specificity of effect.

To inform the mechanism underlying GR rescue of mitotic progression, a series of complementation studies were performed. We engineered domain deletant, phosphodeficient, and transcriptionally compromised GR constructs (Fig. 3A and *SI Appendix*, Fig. S12) and transfected them into a HeLa Flp-In TRex host cell line to generate a panel of eight stable cell lines with doxycycline-regulated GR expression. We used a GR siRNA that targeted the 3' UTR to down-regulate endogenous GR selectively (siRNA11; *SI Appendix*, Fig. S5 B–D) and then rescued GR expression by the addition of doxycycline (Fig. 3B). We again saw a cell cycle phenotype following GR knockdown, further supporting the observations above (Fig. 3C).

GR lacking either the N-terminal sites or phosphosites (Δ AF1GR, S203AGR, and S211AGR) was still efficient at rescuing the cell cycle phenotype (Fig. 3D). Although overexpression of transcriptionally impaired and DNA-binding domain deletant GR (R477C and Δ DBDGR) provided significant rescue, it was

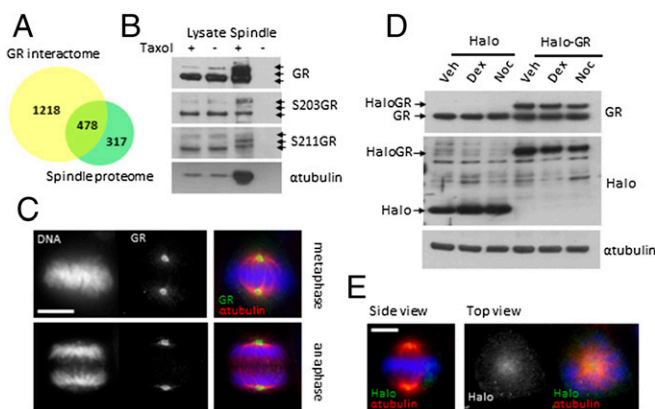


Fig. 1. GR enriches to the mitotic spindle. (A) GR interacting proteins were compared with a published spindle proteome that identifies interaction of GR with a number of components of the mitotic machinery. (B) Mitotic spindles were purified and immunoblotted for α -tubulin, GR, ^PS203GR, and ^PS211GR. (C) HeLa cells were permeabilized and then fixed and immunolabeled for α -tubulin and GR. Metaphase and anaphase cells are shown. (D) HeLa cells were transfected with either Halo or Halo-GR. Twenty-four hours later, cells were treated with vehicle (Veh) or nocodazole (Noc) for 16 h or with 100 nM dexamethasone (Dex) for 1 h and then lysed and immunoblotted for GR, Halo, or α -tubulin. (E) HeLa cells were transfected with Halo-GR, permeabilized, and then fixed and immunolabeled for α -tubulin and Halo. DNA was counterstained with Hoechst. (Scale bars: 10 μ m.)

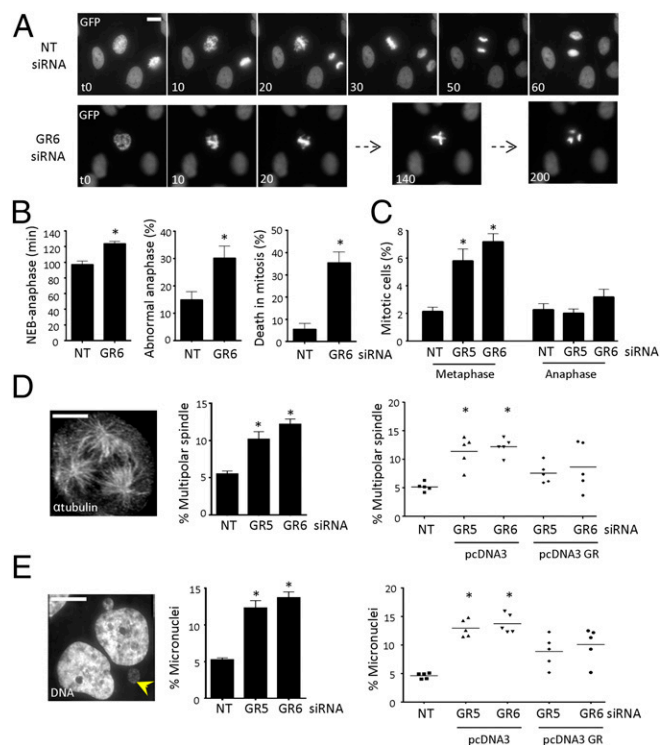


Fig. 2. Reduction of GR expression delays anaphase transition and increases the frequency of chromosomal abnormalities. HeLa cells stably expressing H2B-GFP were transfected with 50 nM nontargeting (NT) or GR-specific (GR6) siRNA, and mitotic events were imaged in real time [values depict minutes from an arbitrary start point (t0)]. Representative fields are shown. (A) Images were scored for average time to completion of mitosis, frequency of abnormal mitotic events, and death in mitosis. (B) Ten fields were tracked per replicate. Graphs show mean \pm SEM from four independent duplicate experiments. *Movies S1–S6* are provided as supplementary information. NEB, nuclear envelope breakdown. (C–E) HeLa cells were treated with 50 nM NT or two different GR-specific (GR5 and GR6) siRNAs, together with siRNA-resistant GR plasmid (pcDNA3GR) or empty vector control (pcDNA3) for 48 h, and then fixed and labeled with an α -tubulin-specific antibody and Hoechst DNA dye counterstain. Cells were imaged and scored for mitotic phase (C) and for evidence of mitotic abnormalities, including spindle deformities (D) and interphase micronuclei (E, yellow arrowhead). Graphs depict mean \pm SEM of at least three independent triplicate experiments. Data were analyzed by the Mann–Whitney test or ANOVA, where $*P < 0.05$. (Scale bars: 10 μ m.)

noted that the efficiency of rescue of Δ DBDGR was less than full-length GR (Fig. 3D).

Only loss of the GR ligand-binding domain (Δ LBDGR) significantly impaired rescue of the mitotic phenotype (impaired rescue was not due to inefficient expression, as shown in Fig. 3D, *Inset*). Interestingly, a point mutation, C736SGR, which renders the GR resistant to endogenous ligands (*SI Appendix, Fig. S12C*), also provided efficient rescue (Fig. 3D), suggesting that ligand binding or GR activation is not necessary for the rescue phenotype but, rather, that the ligand-binding domain may provide the surface for an important protein–protein interaction. We also examined the effect of GR knockdown on a panel of GR target genes under the same experimental conditions. A single transcript of the 15 measured genes showed transcriptional regulation following GR knockdown (*SI Appendix, Fig. S13*), making a transcriptional role for the GR unlikely. These data, coupled with the spindle localization of GR and functional interaction with mitotic proteins and kinases, support the hypothesis that the unliganded GR, and specifically its ligand-binding domain, plays a direct role in regulating mitotic spindle function.

To explore this cell cycle phenotype in vivo, we first analyzed mouse embryonic fibroblasts derived from GR-deficient (GR^{−/−})

mice, which are not viable due to perinatal mortality. Initial analysis of DNA content by fluorescence-activated cell sorting identified no evidence of aneuploidy ($>4n$) in low-passage cells, but chromosome counts demonstrated a trend toward chromosomal aberrations in GR-null cells (29% vs. 22% of cells with a normal chromosome complement in GR^{+/+} and GR^{−/−} cells, respectively; $P = 0.055$). However, GR-null cells showed clear protection from aneuploidy at high passage (Fig. 4A). This complex phenotype may result from GR loss driving cells to death in mitosis, as seen above with acute GR knockdown.

We also investigated mild genotoxic challenge, BrdU treatment, in GR^{+/−} mice (Fig. 4B). Accumulation of dsDNA breaks is a consequence of aberrant chromosome segregation (25) and offers a robust and enduring “mark” of such anomalies. We analyzed livers from BrdU-infused GR^{+/+} and GR^{+/−} mice and found evidence of more dsDNA breaks (γ -H2AX staining) in BrdU-treated GR^{+/+} animals compared with the GR^{+/−} animals (26). We also analyzed aged GR^{+/−} mice (100 wk of age) and found an increased incidence of dsDNA breaks in both the liver and colon (Fig. 4C). In liver, DNA damage was identifiable throughout the tissue, whereas in colon, it appeared to be confined to the epithelial cell layer, which is consistent with sites of the highest GR expression in healthy tissue (*SI Appendix, Fig. S14*).

In our analysis of tissue from aged GR haploinsufficient mice, we found an increase in microtumors in the GR-deficient livers (Fig. 4D) and lungs (Fig. 4E) compared with WT. No differences in premature mortality within the colony were noted, supporting a requirement for additional accumulated genomic damage over time for malignant transformation. We observed that the tumors were further depleted for GR expression compared with surrounding tissue, providing strong evidence linking GR expression with tumorigenesis in vivo (Fig. 4D and E).

Our data suggest that loss of GR expression may contribute to tumorigenesis by impairing accurate chromosome segregation.

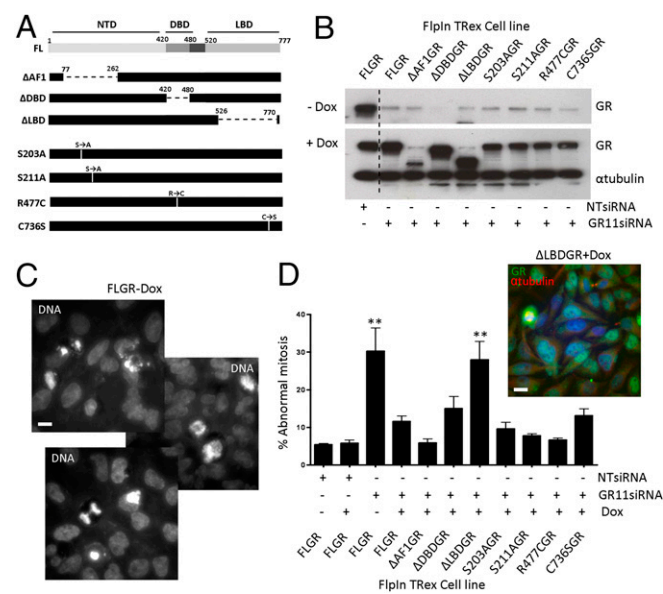


Fig. 3. GR regulation of chromosome segregation requires the GR ligand-binding domain. (A) Schematic showing GR deletant and mutant constructs. Stable HeLa Flp-In TRex-GR cells were transfected with NT or GR-specific siRNA (GR11) and then treated with 5 μ M doxycycline (Dox). Forty-eight hours later, cells were lysed and immunoblotted for GR (B) or fixed and nuclei counterstained with Hoechst (C). (D) Aberrant mitoses were scored and quantified. The graph depicts mean \pm SEM of three independent triplicate experiments. *Inset* Image shows expression of Δ LBDGR. Data were analyzed by ANOVA, where $**P < 0.01$. DBD, DNA-binding domain; FLGR, full-length GR; LBD, ligand-binding domain; NTD, N-terminal domain. (Scale bars: 10 μ m.)

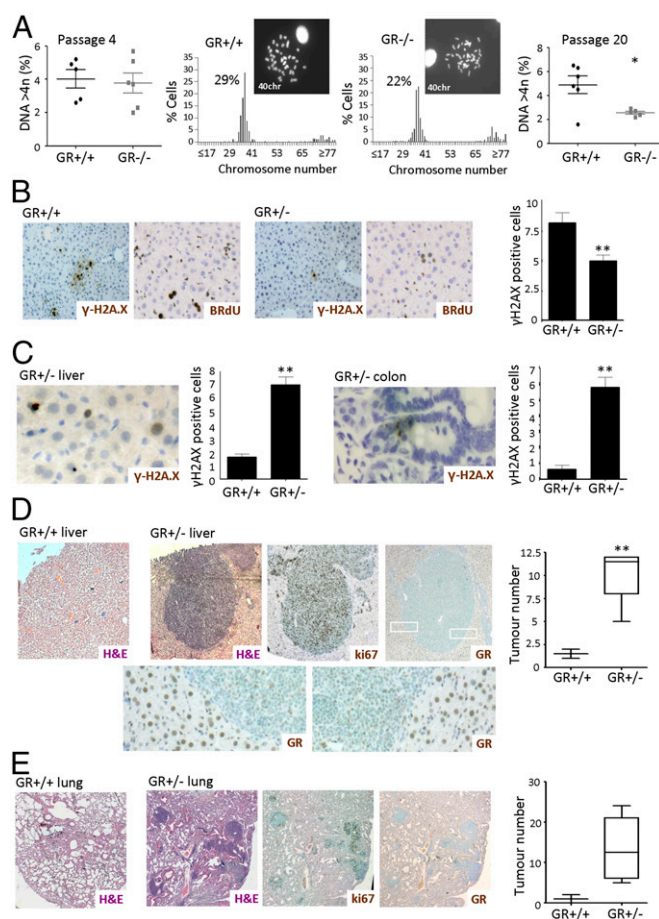


Fig. 4. GR haploinsufficiency increases DNA damage and tumor formation in vivo. (A) Low-passage (p4) GR^{+/+} and GR^{-/-} mouse embryonic fibroblasts (MEFs) were analyzed for DNA content by fluorescence-activated cell sorting (FACS) and also metaphase spread assays. High-passage (p20) GR^{+/+} and GR^{-/-} MEFs were analyzed for DNA content by FACS. (B) Livers from 12-wk-old GR^{+/+} and GR^{-/-} mice infused with BRdU were stained for BRdU or γ-H2AX as an indicator of DNA damage. BRdU or H2A.X staining is shown in brown, and nuclei are counterstained with toluidine blue. A minimum of 1,000 cells for each section were scored from three to four different animals. (C) Liver and colon from 100-wk-old GR^{+/+} and GR^{-/-} mice were analyzed for γ-H2AX as an indicator of DNA damage. A minimum of 10,000 cells were scored for each section from two to four different animals. (D) Immunohistochemistry of serial liver sections from a GR^{+/+} mouse labeled for Ki-67 and GR. (Insets) Higher magnification images are shown. Liver tumors from multiple animals are quantified. (E) Immunohistochemistry of serial lung sections from a GR^{+/+} mouse labeled for Ki-67 and GR. Lung tumors from multiple animals are quantified. The graph shows mean ± SEM values. (Magnification: B, 10×; C, 40×; D and E, 2.5×.) Data were analyzed by the Mann-Whitney test, where *P < 0.05 and **P < 0.01.

To explore this possibility in human cells, we first analyzed B lymphoblasts from patients with GR haploinsufficiency [caused by a frameshift mutation in the GR gene (27)]. Chromosome complement was significantly lower in cells from three haploinsufficient individuals compared with an unaffected family member (Fig. 5A and B and *SI Appendix*, Fig. S15). We also examined GR expression in a comprehensive panel of human cancers and discovered significantly reduced transcript across all tumors (Fig. 5C). We find significant down-regulation of GR transcript in liver, lung, and prostate (Fig. 5D); colon (Fig. 5E); breast (Fig. 5F); and esophageal, ovarian, and endometrial cancers (*SI Appendix*, Fig. S16A). We confirmed loss of GR protein in human colon and breast cancer tissue arrays by immunohistochemistry (Fig. 5G and H and *SI Appendix*, Fig. S16B). Interestingly, we find that the effect

is tissue-specific, with no significant difference in GR expression seen in human bone or gastric cancers (*SI Appendix*, Fig. S17A) and a significant up-regulation of GR transcript seen in pancreatic and renal cancers (*SI Appendix*, Fig. S17B), along with increased GR protein (*SI Appendix*, Fig. S17C).

Even within a single tissue, different tumor subtypes showed variation in GR expression, indicating a major cell type-specific effect (*SI Appendix*, Fig. S18). There were no sex-related differences in GR expression (*SI Appendix*, Fig. S19A) and no correlation between GR and estrogen receptor (ERα) expression across all breast cancer subtypes or with androgen receptor (AR) expression in prostate cancer (*SI Appendix*, Fig. S19B).

Discussion

The GR plays a key role in energy metabolism, immunity, and cell fate determination. Now, our data identify a role in mitotic progression and chromosome segregation. The distribution of GR through metaphase and anaphase overlaps with the spindle centrosome, which plays a major role in coordinating accurate chromosome segregation (28–32). Moreover, under conditions not permissive for GR transcriptional effects, there was a clear biological role, implying a nontranscriptional mechanism of action.

Mitosis is a dynamic process, controlled by the activity of multiple kinases, with attendant modification of proteins facilitating strict checkpoint control. Aurora kinases are master regulators of both centrosome and checkpoint control function. Aurora A is found at centrosomes in early mitosis and controls centrosome maturation, centriole separation, and chromosome alignment (33), whereas accurate targeting of Aurora B to the chromosomes,

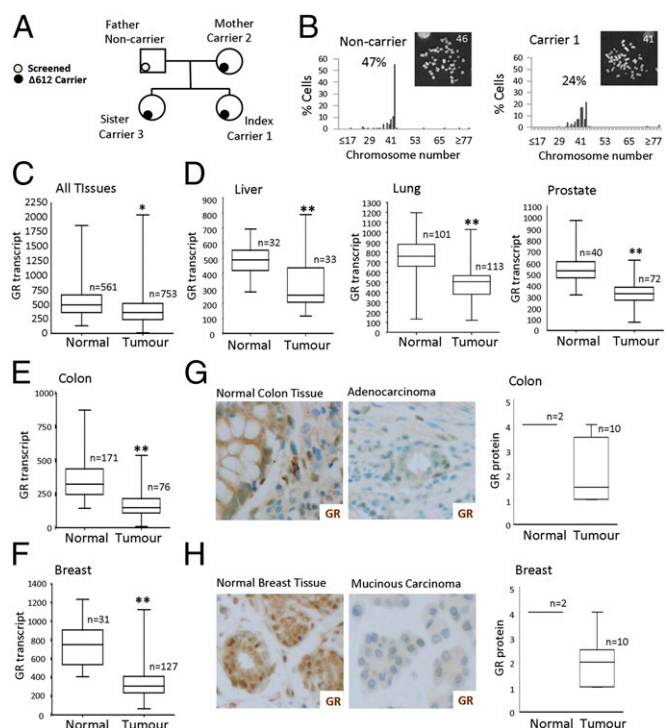


Fig. 5. GR is down-regulated in human cancers. B lymphocytes from a family with GR haploinsufficiency (A) were analyzed for chromosome complement by metaphase spread assay (B). Chromosome counts show counts for 150 cells from three independent passages (p5–p8). (C) Microarrays of tumor and matched normal tissues from 1,314 patients were analyzed for GR transcript. (D) GR transcript in liver, lung, and prostate is shown. GR transcript and protein are shown for colon (E and G) and breast (F and H) cancers. (Magnification: 20×.) Graphs depict median and interquartile range. n, number of patient samples within each group. Data were analyzed by ANOVA, followed by Tukey's correction or the Mann-Whitney test, where *P < 0.05 and **P < 0.001.

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